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A novel ene-reductase from *Synechococcus* sp. PCC 7942 for the asymmetric reduction of alkenes

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ABSTRACT

The increasing demand for enantiopure molecules in the pharmaceutical and fine-chemical industry requires the availability of well-characterized and efficient biocatalysts for asymmetric syntheses. Thereby, asymmetric reduction of alkenes represents one of the most employed reactions for the production of chiral molecules. Here, we present a novel ene-reductase from the cyanobacterium *Synechococcus* sp. PCC 7942, a member of the old yellow enzyme family, capable of reducing C=C bonds in a *anti*-specific fashion. We evaluated its biocatalytic potential by characterizing the substrate spectrum, cofactor preference, stereoselectivity and biochemical properties. This NADPH-dependent flavoprotein accepted a wide range of activated alkenes and displayed a pH optimum between pH 7.6 and pH 8.6. A C-terminal His_6-tag decreased the enzyme activity 2.7-fold, but did not influence the stereoselectivity. The reduction of (R)-carvone and 2-methylmaleimide yielded (R)-products with high optical purities (98% de and >99% ee, respectively), pointing out the applicability of this new biocatalyst in the stereoselective production of chiral compounds.

1. Introduction

Over 50% of current drug candidates are developed as single enantiomers, highlighting the increasing demand of the pharmaceutical industry for enantiopure compounds. Hence, the industry has to meet the challenges of providing valuable chiral building blocks or adequate toolboxes for the synthesis of chiral materials [1,2].

Asymmetric reduction of C=C bonds represents one of the most widely employed strategies for the production of chiral molecules, because it leads to the creation of up to two stereogenic centres [3–5]. Whereas *syn*-hydrogenation has reached an industrial standard using transition metal-based homogeneous catalysts [6,7], *anti*-hydrogenation is still at a development stage [8]. In this field, biocatalysis can offer great opportunities to overcome the limitations of chemical synthesis, as well as provide economical and ecological benefits [9,10].

Anti hydrogen addition can be performed using ene-reductases (ER) [EC 1.3.1.X], which are NAD(P)H and flavin-dependent enzymes of the old yellow enzyme (OYE) family. The substrate spectra of these enzymes are remarkably broad ranging from α,β-unsaturated aldehydes, ketones, imides to nitriles, carboxylic acids and nitro aromatics [3,11]. The reaction proceeds by a bi–bi ping pong mechanism consisting of the hydride transfer from NAD(P)H to the FMN cofactor, followed by the reduction of the activated alkene by a hydride transfer from FMN [12–14].

Since the discovery of OYE in baker's yeast in 1933 [15], numerous OYE homologues have been identified in bacteria, yeast, fungi and plants [16–23]. Although these enzymes show similarities with regard to their substrate spectra [3], variations in enzyme activity, cofactor preference [19,24] and stereoselectivity [4,17,25,26] promote the search for new enzymes.

Cyanobacteria represent one of the largest sub-groups of gram-negative prokaryotes [27] and have received widespread attention in recent years due to their capability in the biofuel production [28]. Though cyanobacterial species are known as a valuable source for novel bioactive compounds including therapeutics, insecticides and anti-fouling agents [29], efforts regarding the investigation of new useful enzymes from cyanobacteria remain sparsely [30,31]. Whole-cell biotransformations of enones were reported using the freshwater cyanobacterium *Synecococcus* sp. PCC 7942 yielding excellent stereoselectivities [32,33]. However, the efficiency of biotransformations using cyanobacteria was limited by the formation of by-products (alcohols) and long reaction times [32]. Thus, here we present a novel recombinant ene-reductase from *Synechococcus* sp. PCC 7942 for the asymmetric synthesis of valuable enantiopure materials.

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2. Materials and methods

2.1. Chemicals and enzymes

Chemicals and solvents were of analytical grade from commercial sources. Substrates and reference materials were obtained from Sigma–Aldrich (Schnelldorf, Germany). 2-Methylmaleimide and *rac*-2-methylsuccinimide were synthesized as reported previously [26,34]. NMR spectra were in accordance with those reported in the literature [26]. NADH and NADPH were purchased from Carl Roth (Karlsruhe, Germany). Enzymes used for DNA manipulations were bought from New England Biolabs (Frankfurt, Germany). Primers were synthesized by Metabion (Martinsried, Germany).

2.2. Cloning

The DNA sequence of the ene-reductase from *Synechoccocus* sp. PCC 7942 was obtained from the NCBI database (RefSeq ID: NC_007604). Genomic DNA from *Synechoccocus* sp. PCC 7942 (Pasteur Culture Collection of cyanobacteria, Paris, France) was used as template for the amplification of the ER gene. The PCR was performed using Phusion® HF DNA polymerase (forward primer, pET21a(+): 5′-AGAGATCATATGTCCGAATCGCTCAAACTGCTGACG-3′; reverse primer, pET21a(+): 5′-AGAGATGCGGCCGCGACAGATGCTGCTTCCAAACTGGGATAG-3′; forward primer, pETM-41: 5′-AGAGATCCATGGACATGTCCGAATCGCTCAAACTGCTGACG-3′; reverse primer, pETM-41: 5′-AGAGATGCGGCCGCTTAGACAGATGCTGCTTCCAAACTGGGA-3′). The ER gene was integrated into pET21a(+) (Novagen, San Diego, USA) using the restriction sites NdeI and NotI for a construct with a C-terminal His_6-tag (Syn7942ER-His_6), as well as into pETM-41 (EMBL, Heidelberg, Germany) using NcoI and NotI. The vector pETM41 contained an N-terminal His_6-maltose-binding-protein (His_6-MBP) tag (His_6-MBP-Syn7942ER), which was removed using the tobacco etch virus (TEV) protease cleavage site to obtain the enzyme without any tags (Syn7942ER). The constructed vectors were transformed into competent *Escherichia coli* (*E. coli*) DH5α (Invitrogen, Carlsbad, CA, USA). After confirming the accuracy of the constructed vector by sequencing (GATC, Konstanz, Germany), plasmids were transformed into *E. coli* BL21(DE3) (Novagen, San Diego, USA) for protein expression.

2.3. Recombinant expression and purification

A 5 mL Terrific Broth (TB) preculture supplemented with the respective antibiotics (50 mg L^{-1} ampicillin for pET21a(+) and 34 mg L^{-1} kanamycin for pETM-41) was inoculated with a single colony from an agar plate, incubated over night at 37 °C and then subcultured into 200 mL TB in 1000 mL shake flasks without baffles. As the cell density reached an OD_{600} of 0.6–0.8, the protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Afterwards, cells were incubated overnight (18–20 h) at 20 °C and 160 rpm. After harvesting cells by centrifugation, pellets were stored at −20 °C or used directly for protein purification.

TEV protease, His_6-MBP-Syn7942ER fusion protein and Syn7942ER-His_6 were purified according to a modified protocol described by Hölsch et al. [30]. Cell pellets were suspended in binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 40 mM imidazole, pH 7.4) and disrupted using 50% (v/v) glass beads (0.25–0.5 mm; Carl Roth, Karlsruhe, Germany) for 3 min at 30 Hz in a mixer mill (Retsch, Haan, Germany). After centrifugation (47,808 × *g*, 4 °C, 30 min), the supernatant was filtered (0.45 μm, Minisart HF, Sartorius Stedim Biotech, Göttingen, Germany) and applied to a 1 mL HisTrap FF crude column (GE Healthcare, Uppsala, Sweden). Buffer exchange and concentration of purified proteins were performed using a Vivaspin ultrafiltration device (molecular weight cutoff: 5 kDa; Sartorius Stedim Biotech, Göttingen, Germany).

2.4. Protein analysis

Protein concentration was determined using the BCA Protein Assay (Thermo Fisher Scientific, Rockford, USA). SDS-PAGE was performed using 3% and 12.5% Bis–Tris gels in Tris–glycine running buffer with Roti®-Mark Standard (Carl Roth, Karlsruhe, Germany) for the estimation of the molecular mass and protein purity. Gels were stained according to Fairbanks et al. [35].

2.5. Enzyme assay

The enzyme activity was determined by a photometric assay monitoring the oxidation of NADPH concentration at 340 nm using a molar absorption coefficient of 6.22 mM^{-1} cm^{-1}. In case of ketoisophorone and 3-phenyl-2-methylpropenal the assay was performed at 365 nm using a molar absorption coefficient of 3.51 mM^{-1} cm^{-1}. All reactions were performed on a 200 μL scale in sodium phosphate buffer (100 mM, pH 7.0) at 30 °C or 25 °C using microplate spectrometers (EL808, BioTek Instruments, Winooski, USA/infinite M200, Tecan, Crailsheim, Germany).

All assays contained 10 mM maleimide and 0.5 mM NADPH, unless stated otherwise. Buffers for the investigation of the pH profile were 50 mM sodium citrate, 50 mM sodium phosphate, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and 50 mM Tris–HCl applied in a pH range of pH 5.5–9.5. The effect of organic solvents was studied using ethanol, 1-propanol, iso-propanol and dimethylformamide (DMF). For the determination of kinetic parameters, 10 mM maleimide were kept constant, whereas the concentration of NADPH was varied between 0.01 mM and 0.6 mM. Kinetic parameters were estimated according to the Michaelis–Menten equation [36] and data were analyzed using non-linear regression analysis (Sigma Plot 8.0, SPSS, Chicago, USA). Investigation of the substrate spectrum was conducted with 0.5 mM NADPH using substrates at a concentration of 10 mM (added as an ethanol solution, <5% (v/v) final ethanol concentration). Appropriate controls were included and experiments were conducted at least in triplicates. One unit of enzyme activity was defined as the oxidation of 1 μmol NADPH per minute. Background oxidase activity was determined and subtracted from the specific activity.

2.6. Bioreduction on a mL-scale

The bioreduction of alkenes was carried out in 1 mL sodium phosphate buffer (100 mM, pH 7.0) containing 5 mM substrates (added as a DMF solution, <2% (v/v) final DMF concentration), 15 mM NADH and 85 μg mL^{-1} (2.1 μM) Syn7942ER. The reactions were agitated at 30 °C and 300 rpm (Thermomixer comfort, Eppendorf, Hamburg, Germany) for 24 h. The reduction of ketoisophorone with 5–20% (v/v) ethanol, iso-propanol or DMF was performed on a 1 mL-scale with 57 μg mL^{-1} (1.2 μM) $NADP^+$-dependent *Myc*FDH C145S/D221Q/C255V [37], 250 mM sodium formate, 0.5 mM $NADP^+$, 10 mM ketoisophorone and 25 μg mL^{-1} (0.6 μM) Syn7942ER for 6 h at 30 °C and 150 rpm (WiseCube, Witeg Labortechnik, Wertheim, Germany). Reactions were stopped by extraction with ethyl acetate (1:1) containing 36 mM (R)-limonene as internal standard.

2.7. Analytical procedures

Analysis of conversion and enantiomeric excess was performed using a Varian CP-3800 gas chromatograph (GC) equipped with a flame ionization detector (FID). An Astec Chiraldex B-TA column (40 m, 0.25 mm, Sigma–Aldrich, Schnelldorf, Germany) was applied for the analysis of (R)-carvone, 2-methylmaleimide, 2-methyl-2-cyclopenten-1-one, 3-methyl-2-cyclohexen-1-one and the respective reduction products as described previously [4,17,25]. Ketoisophorone and (R)-Levodione were determined on a CP-Chirasil-DEX CB column (25 m, 0.32 mm, Agilent Technologies, Böblingen, Germany) according to [4]. The absolute configuration of products was identified by comparison with reference materials on chiral GC according to the literature [4,17,25].

3. Results

3.1. Sequence analysis

The ene-reductase from the cyanobacterium *Synechococcus* sp. PCC 7942 (RefSeq ID: YP_399492) was identified using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) databank [38]. The amino acid sequence exhibited a high similarity to OPR1 from *Oryza sativa* (51% identity, 66% similarity) and NemR from *E. coli* (49% identity, 62% similarity). In contrast, the similarity to OYE1 from *Saccharomyces carlsbergensis* and OYE2, OYE3 from *Saccharomyces cerevisiae* was only moderate (40–42% identity, 56–57% similarity). A sequence alignment with other OYE homologues (Fig. 1) demonstrated that Syn7942ER belongs to the so-called "classical ERs" according to the classification by Toogood et al. [3]. In addition, Syn7942ER is considered to be a monomeric protein based on the P-[LM]-T-R-X-R pattern in the loop β1 region and the G-[FYW]-X(3)-P-G-[ILV]-[FHYW] pattern in the loop β2 region according to Oberdorfer et al. [39].

3.2. Cloning, protein expression and purification

Cloning of the ER gene enabled the expression of a protein with a C-terminal His_6-tag (Syn7942ER-His_6) as well as a native protein without an affinity tag (Syn7942ER). Typical yields after protein purification were about 5.6 mg protein per gram dry cell weight (mg g_{DCW}^{-1}) for Syn7942ER-His_6 and 0.5 mg g_{DCW}^{-1} for Syn7942ER. A SDS-PAGE analysis of purified Syn7942ER is shown in Fig. 2. Purified enzymes were used for the characterization of the substrate spectrum, cofactor preference and stereoselectivity,

```
                   1                                                                                              90
         Syn7942ER            MSESLKLLTP VQVGRYELRN RIVMAPLTRN RATGPDNIPN -DLNVLYYQQ RAS--AGLII TEASQISPQG QGYPLTPGIH
             PETNR             MSAEKLFTP LKVGAVTAPN RVFMAPLTRL RSIEPGDIPT -PLMGEYYRQ RAS--AGLII SEATQISAQA KGYAGAPGLH
Group 1       OYE1 MSFVKDFKPQ ALGDTNLFKP IKIGNNELLH RAVIPPLTRM RALHPGNIPN RDWAVEYYTQ RAQRPGTMII TEGAFISPQA GGYDNAPGVW
              OYE3 MPFVKGFEPI SLRDTNLFEP IKIGNTQLAH RAVMPPLTRM RATHPGNIPN KEWAAVYYGQ RAQRPGTMII TEGTFISPQA GGYDNAPGIW
---------------------------------------------------------------------------------------------------------------------
Group 2       XenA               MSALFEP YTLKDVTLRN RIAIPPMCQY MA--EDGLIN -DWHQVHYAS MARGGAGLLV VEATAVAPEG RITPGCAGIW
              Yqjm              MARKLFTP ITIKDMTLKN RIVMSPMCMY SSHEKDGKLT -PFHMAHYIS RAIGQVGLII VEASAVNPQG RITDQDLGIW
                                                         *

                   91                                                                                            180
         Syn7942ER SPEQVEGWKP IVQAVHDRGG CIFLQLWHVG RISHPSLQPD GALPVAPSAI QPAGMAATFQ GEQPFV---- -TPRALETEE IAGIVEDYRR
             PETNR SPEQIAAWKK ITAGVHAEDG RIAVQLWHTG RISHSSIQPG GQAPVSASAL NANTRTSLRD ENGNAIRVDT TTPRALELDE IPGIVNDFRQ
Group 1       OYE1 SEEQMVEWTK IFNAIHEKKS FVWVQLWVLG WAAFPDNLAR DGLRYDSASD NVFMDAEQEA KAKKANN--- -PQHSLTKDE IKQYIKEYVQ
              OYE3 SDEQVAEWKN IFLAIHDCQS FAWVQLWSLG WASFPDVLAR DGLRYDCASD RVYMNATLQE KAKDANN--- -LEHSLTKDD IKQYIKDYIH
---------------------------------------------------------------------------------------------------------------------
Group 2       XenA SDAHAQAFVP VVQAIKAAGS VPGIQIAHAG RKASANRPWE GDDHIGADDA RGWETIAPSA IAFGAHL--P NVPRAMTLDD IARVKQDFVD
              Yqjm SDEHIEGFAK LTEQVKEQGS KIGIQLAHAG RKAEL----E GD-------- ----IFAPSA IAFDEQS--A T-PVEMSAEK VKETVQEFKQ
                                               *

                   181                                                                                           270
         Syn7942ER AAENALAAGF DGVEVHGANG YLIDQFLQDG TNQRSDRYGG SFENRSRFLR EVLDAVISVW GSDRVGLRLS PWGQFNDMRD SDPVGLFS--
             PETNR AVANAREAGF DLVELHSAHG YLLHQFLSPS SNQRTDQYGG SVENRARLVL EVVDAVCNEW SADRIGIRVS PIGTFQNVDN GPNEEADA--
Group 1       OYE1 AAKNSIAAGA DGVEIHSANG YLLNQFLDPH SNTRTDEYGG SIENRARFTL EVVDALVEAI GHEKVGLRLS PYGVFNSMSG GAETGIVAQY
              OYE3 AAKNSIAAGA DGVEIHSANG YLLNQFLDPH SNKRTDEYGG TIENRARFTL EVVDALIETI GPERVGLRLS PYGTFNSMSG GAEPGIIAQY
---------------------------------------------------------------------------------------------------------------------
Group 2       XenA AARRARDAGF EWIELHFAHG YLGQSFFSEH SNKRTDAYGG SFDNRSRFLL ETLAAVREVW PENLPLTARF GVLEYDGRDE QTLEESIE--
              Yqjm AAARAKEAGF DVIEIHAAHG YLIHEFLSPL SNHRTDEYGG SPENRYRFLR EIIDEVKQVW DG--PLFVRV SASDYTDKGL D-IADHIG--
                                   *  *  *

                   271                                                                                           360
         Syn7942ER -YVAQMLNPY N-----LAYL HWIEPRWD-- -KAEESPEFN QMATPVFRSL YNGPVIAAGG YSRS-TAEAA IASGAADLVA FGRLYISNPD
             PETNR LYLIEELAKR G-----IAYL HMSET--D-- -LAGGKP-YS EAFRQKVRER FHGVIIGAGA YTAE-KAEDL IGKGLIDAVA FGRDYIANPD
Group 1       OYE1 AYVAGELEKR AKAGKRLAFV HLVEPRVTNP FLTEGEGEYE GGSNDFVYSI WKGPVIRAGN FALHPEVVRE EVKDKRTLIG YGRFFISNPD
              OYE3 SYVLGELEKR AKAGKRLAFV HLVEPRVTDP SLVEGEGEYS EGTNDFAYSI WKGPIIRAGN YALHPEVVRE QVKDPRTLIG YGRFFISNPD
---------------------------------------------------------------------------------------------------------------------
Group 2       XenA --LARRFKAG G-----LDLL SVSVGFTIPE TNIPWGPAFM GPIAERVRRE AKLPVTSAWG FGTPQLAEAA LQANQLDLVS VGRAHLADPH
              Yqjm --FAKWMKEQ G-----VDLI DCSSGALV-H ADINVFPGYQ VSFAEKIREQ ADMATGAVGM ITDGSMAEEI LQNGRADLIF IGRELLRDPF

                   361                                          404
         Syn7942ER LVERFALDAP LNPYDRNTFY GGDEHGYTDY PSLEAASV
             PETNR LVARLQKKAE LNPQRPESFY GGGAEGYTDY PSL
Group 1       OYE1 LVDRLEKGLP LNKYDRDTFY QMSAHGYIDY PTYEEALKLG WDKK
              OYE3 LVYRLEEGLP LNKYDRSTFY TMSAEGYTDY PTYEEAVDLG WNKN
---------------------------------------------------------------------------------------------------------------------
Group 2       XenA WAYFAAKELG VEKASW-TLP APYAHWLERY R
              Yqjm FARTAAKQLN TE------IP APVQYERGW
                                      *
```

Fig. 1. Amino acid sequence alignment of Syn7942ER and known ERs from the literature. Sequence alignment was performed using Multalin [55] ERs and NCBI accession numbers were: Syn7942ER from *Synechococcus* sp. PCC 7942 (YP_399492), PETNR from *Enterobacter cloacae* st. PB2 (AAB38683), OYE1 from *Saccharomyces carlsbergensis* (Q02899), OYE3 from *Saccharomyces cerevisiae* (CAA97878), XenA from *Pseudomonas putida* (AAF02538) and Yqjm from *Bacillus subtilis* (P54550). ERs were classified into "classical ERs" (Group 1) and "thermophilic-like ERs" (Group 2) according to Toogood et al. [3]. The conservation of active site residues (T38, W117, H197, N200, Y202, Y381, OYE1 numbering) was highlighted (*) and the sequence patterns indicating the quaternary structure (white letters) were shown according to Oberdorfer et al. [39].

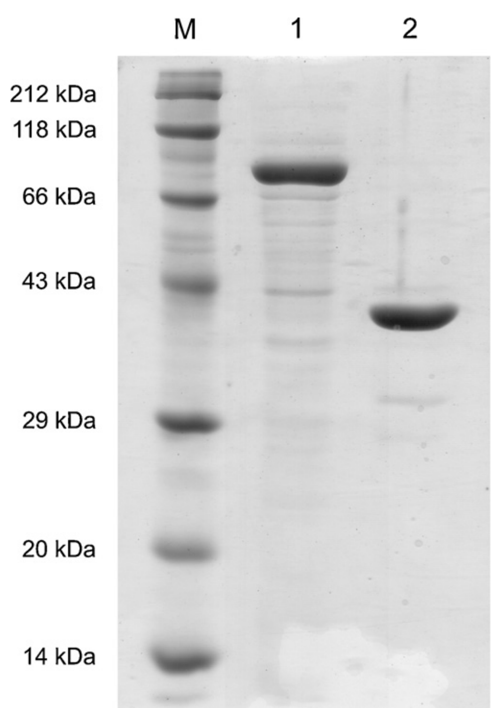


Fig. 2. SDS-PAGE analysis of purified Syn7942ER. Lane M: molecular weight marker, lane 1: crude extract, and lane 2: purified Syn7942ER (after cleavage of the His_6-maltose-binding-protein tag).

as well as the enzyme properties with regard to the effect of temperature, pH and organic solvents.

3.3. Substrate spectrum

The substrate spectrum of Syn7942ER was studied using 22 alkenes from different substance classes (Table 1). Syn7942ER showed high activities for maleimide derivatives (1.35–2.37 $U\,mg^{-1}$). In addition, cyclic enones were accepted with good rates (0.26–1.16 $U\,mg^{-1}$). Ketoisophorone, a molecule possessing two carbonyl groups, led to a higher activity (1.16 $U\,mg^{-1}$) compared to enones with one carbonyl group (<0.75 $U\,mg^{-1}$). In contrast to 2-cyclohexen-1-one, a 2.9-fold lower reaction rate was shown for the enone with a smaller ring size (2-cyclopenten-1-one, 0.26 $U\,mg^{-1}$). Syn7942ER accepted (R)- and (S)-carvone with a slight preference for the (R)-enantiomer (0.61 $U\,mg^{-1}$ and 0.37 $U\,mg^{-1}$, respectively). Both carbonyl and nitro groups were accepted equally as the electron-withdrawing activating group (0.75 $U\,mg^{-1}$ for 2-cyclohexen-1-one compared to 0.83 $U\,mg^{-1}$ for 1-nitrocyclohexene). No enzyme activity was detected using β-substituted cyclic enones (3-methyl-2-cyclopenten-1-one, 3-methyl-2-cyclohexen-1-one), enones with exocyclic C=C bonds ((S)-/(R)-pulegone) and dicarboxylic acids (itaconic acid, mesaconic acid).

The substrate spectrum was also investigated for Syn7942ER-His_6 with all 22 compounds. The C-terminal His_6-tag resulted in an overall reduction of the specific activity by an average factor of 2.7 ± 0.9, though it did not influence the preference for specific substrate types (data not shown).

3.4. Cofactor specificity and kinetic parameters

Since the preference for the cofactors NADPH and NADH differs within the members of the OYE family [24], the cofactor specificity of the Syn7942ER was investigated. Syn7942ER preferred NADPH over NADH with a ratio of 22:1. The apparent K_m and ν_{max} for NADPH with maleimide as substrate were 0.36 ± 0.06 mM and 2.80 ± 0.29 U mg^{-1}, respectively. The C-terminal His_6-tag did not change the cofactor preference (NADPH:NADH ratio: 18:1), but it had a substantial impact on the apparent kinetic constants for NADPH, lowering the ν_{max} 3.1-fold and the K_m 3.6-fold.

Table 1
Substrate spectrum of Syn7942ER. Specific activities were determined at 25 °C using 10 mM substrate and 0.5 mM NADPH. nd: not detected.

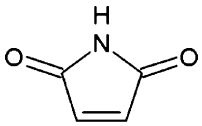
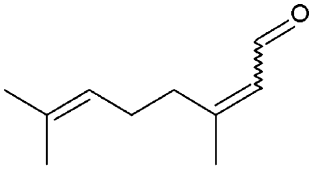
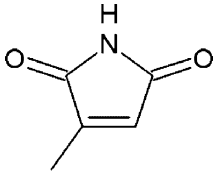
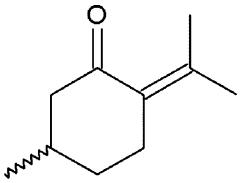
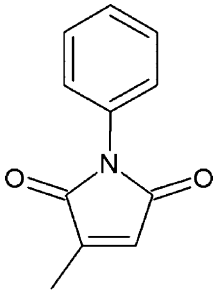
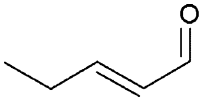
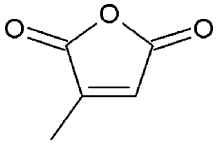
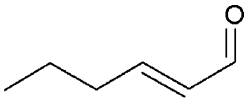
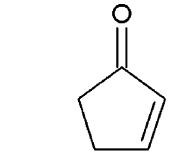
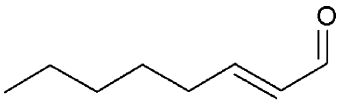
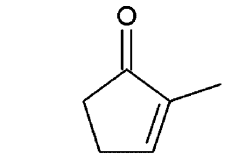
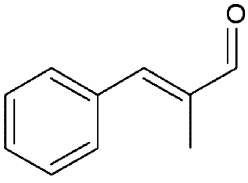
Substrate	Specific activity (U mg^{-1})	Substrate	Specific activity (U mg^{-1})
 maleimide	1.35 ± 0.16	 citral (cis/trans)	nd
 2-methylmaleimide	2.30 ± 0.20	 (R) or (S)-pulegone	nd
 2-methyl-*N*-phenylmaleimide	1.61 ± 0.12	 *trans*-2-penten-1-al	1.31 ± 0.09
 citraconic anhydride	nd	 *trans*-2-hexen-1-al	1.57 ± 0.10
 2-cyclopenten-1-one	0.26 ± 0.13	 *trans*-2-octen-1-al	1.45 ± 0.16
 2-methyl-2-cyclopenten-1-one	nd	 3-phenyl-2-methylpropenal	0.19 ± 0.01

Table 1 (*Continued*)

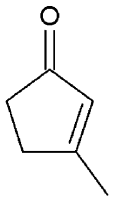
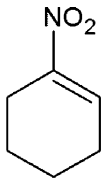
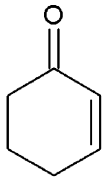
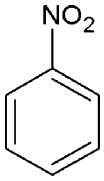
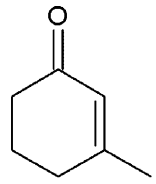
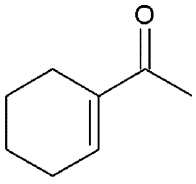
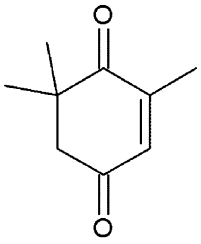
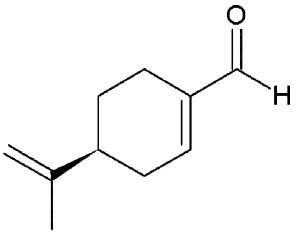
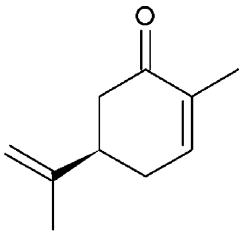
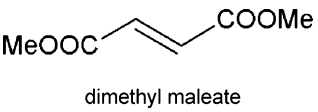
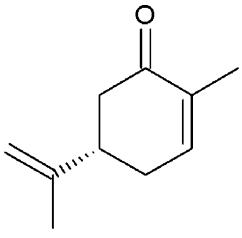
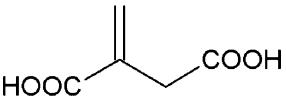
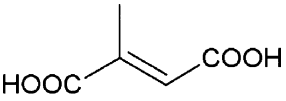
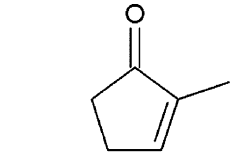
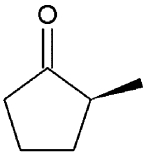
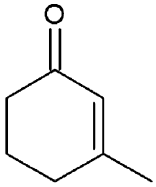
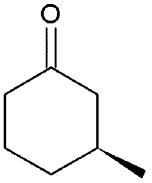
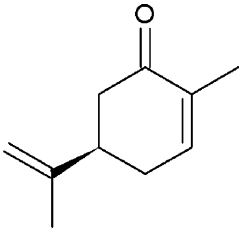
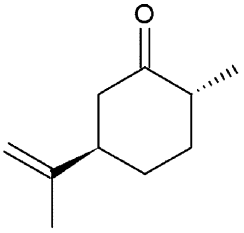
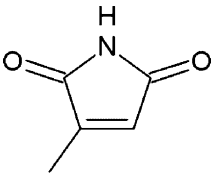
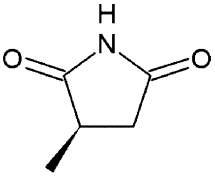
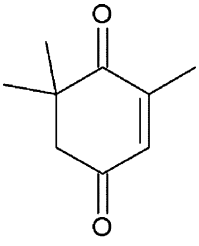
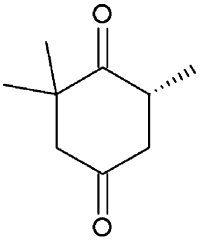
Substrate	Specific activity (U mg^{-1})	Substrate	Specific activity (U mg^{-1})
 3-methyl-2-cyclopenten-1-one	nd	 1-nitrocyclohexene	0.83 ± 0.03
 2-cyclohexen-1-one	0.75 ± 0.03	 nitrobenzene	nd
 3-methyl-2-cyclohexen-1-	nd	 1-acetyl-1-cyclohexene	nd
 ketoisophorone	1.16 ± 0.02	 (S)-perillaldehyde	0.52 ± 0.06
 (R)-carvone	0.61 ± 0.18	 dimethyl maleate	0.55 ± 0.02
 (S)-carvone	0.37 ± 0.10	 itaconic acid	nd
		 mesaconic acid	nd

Table 2
Bioreduction with Syn7942ER. Assays contained 86 μg mL^{-1} (2.1 μM) purified ER, 15 mM NADH and 5 mM substrates (2-methyl-2-cyclopenten-1-one, 3-methyl-2-cyclohexen-1-one, (R)-carvone, 2-methylmaleimide and ketoisophorone). Reactions were performed for 24 h at 30 °C. Conversions (Conv., %) and enantiomeric excesses (ee, %)/diastereomeric excesses (de, %) were determined by chiral GC.

Substrate	Product	Conv. (%)	ee/de (%)
 2-methyl-2-cyclopenten-1-one	 (S)-2-methylcyclopentanone	70	76 ee
 3-methyl-2-cyclohexen-1-one	 (S)-3-methylcyclohexanone	2	72 ee
 (R)-carvone	 (2R,5R)-dihydrocarvone	>99	99 ee/98 de
 2-methylmaleimide	 2-methylsuccinimide	>99	99 ee
 ketoisophorone	 (R)-levodione	>99	17ee
		93 (3 h)	97ee (3 h)

3.5. Asymmetric reduction

The stereoselectivity of Syn7942ER was evaluated using 2-methyl-2-cyclopenten-1-one, 3-methyl-2-cyclohexen-1-one, (R)-carvone, 2-methylmaleimide and ketoisophorone (Table 2). The five substrates were chosen as representatives for the substance classes: maleimide derivatives, terpenoids and enones. In addition, both (R)-carvone and ketoisophorone are commercially interesting prochiral substrates.

Syn7942ER reduced 2-methylmaleimide with high rate and stereoselectivity forming (R)-methylsuccinimide (>99% conversion and 99% enantiomeric excess (ee)). (R)-carvone was converted in a *anti*-fashion yielding (2R,5R)-dihydrocarvone with an excellent stereoselectivity of 98% diastereomeric excess (de). Reduction of 2-methyl-2-cyclopenten-1-one resulted in the (S)-enantiomer with low-to-modest optical purity (76% ee). In case of ketoisophorone, the reduction over 24 h furnished (R)-levodione with a low enantiomeric excess (17% ee). The low enantiopurity was caused by product racemization in aqueous solutions [17], given that molecules with a stereogenic centre at the Cα position are prone to racemise [40–42]. This was supported by the fact that the reduction of the enantiomeric excess occured after a reaction time of 3 h, reaching 97% ee at 93% conversion of ketoisophorone. No significant influence of the C-terminal His_6-tag was observed on the

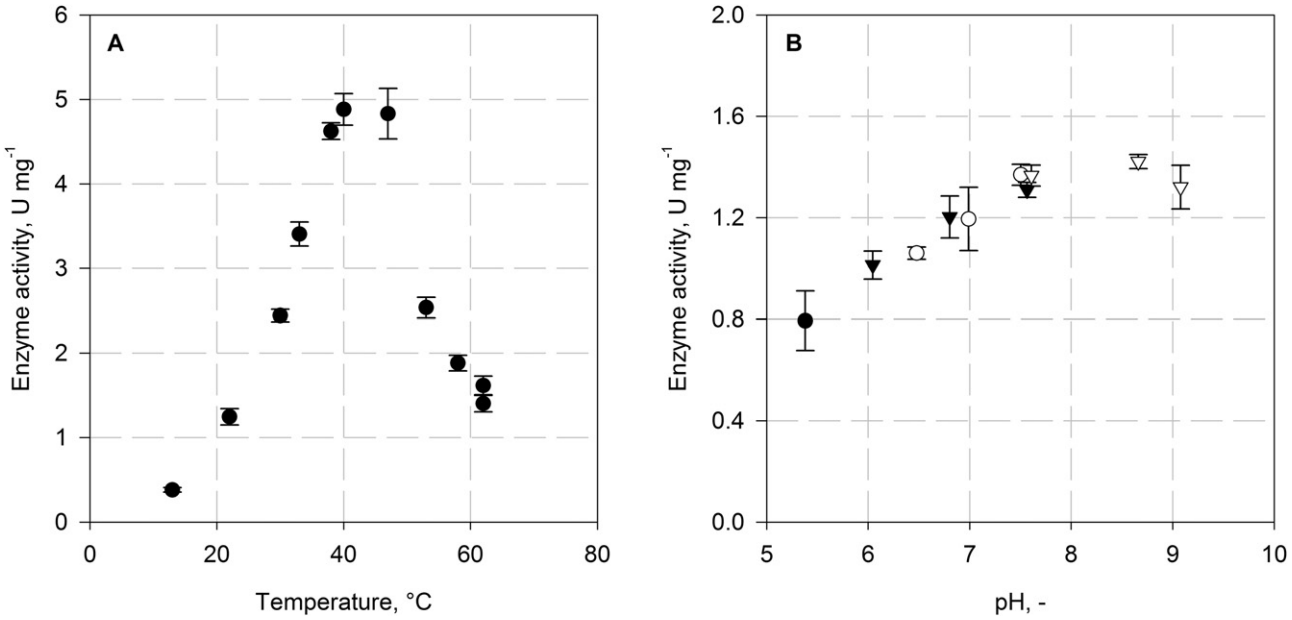


Fig. 3. Temperature and pH optima of Syn7942ER. (A) Effect of temperature on the enzyme activity of Syn7942ER. (B) pH profile of Syn7942ER using 50 mM sodium citrate (black circle), 50 mM sodium phosphate (white circle), 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (black triangle), and 50 mM Tris–HCl (white triangle). Reactions were performed with 10 mM maleimide and 0.5 mM NADPH.

stereoselectivity of Syn7942ER. Thus, the C-terminal His_6-tag did not seem to impact the binding mode of substrates prior to the hydride transfer from FMN.

3.6. Temperature and pH optima

Syn7942ER was characterized with regard to the effect of temperature and pH on the enzyme activity using maleimide as substrate (Fig. 3). A broad temperature optimum was determined ranging from 40 °C to 47 °C. An activation energy of 63.76 kJ mol^{-1} was calculated following the Arrhenius equation. The pH optimum for Syn7942ER was identified between pH 7.6 and pH 8.6 with the highest activity achieved using Tris–HCl buffer.

3.7. Effect of salts and organic solvents

The effect of salts and co-solvents on the reaction rate of Syn7942ER was studied in assays with maleimide. The enzyme activity was not significantly influenced by concentrations of up to 0.3 M sodium phosphate (data not shown) and 1 M sodium chloride (Fig. 4A). However, 3 M sodium chloride resulted in a reduction of the enzyme activity by 31%. The effect of water miscible solvents on the enzyme activity of Syn7942ER was investigated using ethanol, 1-propanol, isopropanol and DMF (Fig. 4B). Ethanol appeared to be the best solvent, since the enzyme activity was not impaired by 5% (v/v) ethanol and 80% of the enzyme activity was retained in the presence of 20% (v/v) ethanol. In contrast, 20% (v/v) DMF led to a reduction of the enzyme activity by 75%.

In addition, the catalytic performance of Syn7942ER was investigated in the presence of 5–20% ethanol, iso-propanol and DMF during the bioreduction of ketoisophorone in mL-scale (Fig. 5). Increasing amounts of organic solvents resulted in lower conversions. The highest conversions were achieved using 5–10% (v/v) ethanol and iso-propanol. At 20% (v/v), the reaction with iso-propanol yielded a 17% lower conversion compared to ethanol. In contrast, conversions were reduced by 13–23% at the same concentrations of DMF compared to isopropanol and ethanol, which is consistent with the results of the activitiy assays. Therefore, the enzymatic reaction rate is determined by the concentration and choice of the organic solvent, which can impair the catalytic structure and enzyme stability [9,43]. Syn7942ER exhibited a high stereoselectivity in the formation of (R)-levodione, if the non-enzymatic racemisation of the product in aqueous medium is minimized (Table 2). The results indicated that the enantiopurity of (R)-levodione decreased with higher conversions. Fryszkowska et al. [17] showed that the enantiomeric excess of (R)-levodione depends on the relative rates of the enzymatic reaction and the non-enzymatic racemisation. When the reaction rate decreases with increasing conversion, the racemisation process increases proportionally to the product concentration (first-order reaction), resulting in lower enantiopurities at higher conversions. The reactions with DMF resulted in significant lower stereoselectivities at similar conversions compared to the reactions with ethanol and iso-propanol. This result indicates that organic solvents might also influence the stereoselectivity of the enzyme or the water-mediated racemisation [9,43].

4. Discussion

Biocatalysis is considered as a valuable option for the stereoselective reduction of activated C=C bonds. Not only molecules with high optical purities can be produced using ERs, also the stereochemical outcome of the reaction can be controlled by the choice of substrate or enzyme [4,44–46]. Although ERs show similarities regarding the stereochemistry of the product, both reaction rates and optical purities of the product vary between homologous enzymes [3]. This promotes the search for new biocatalysts performing highly stereoselective reactions.

In this paper, we have characterized a novel ER from *Synechococcus* sp. PCC 7942 with respect to its biocatalytic potential for the asymmetric hydrogenation. The impact of a C-terminal His_6-tag, which is useful for convenient protein purification, was studied. A significant reduction in enzyme activity was observed for Syn7942ER-His_6 without a clear preference for a specific substrate type. The K_m for NADPH was increased 3.6-fold, whereas the stereoselectivity was not influenced. Therefore, the affinity tag did not seem to alter the binding mode of alkenes, but probably the hydride transfer from NADPH to FMN. Strong structural and

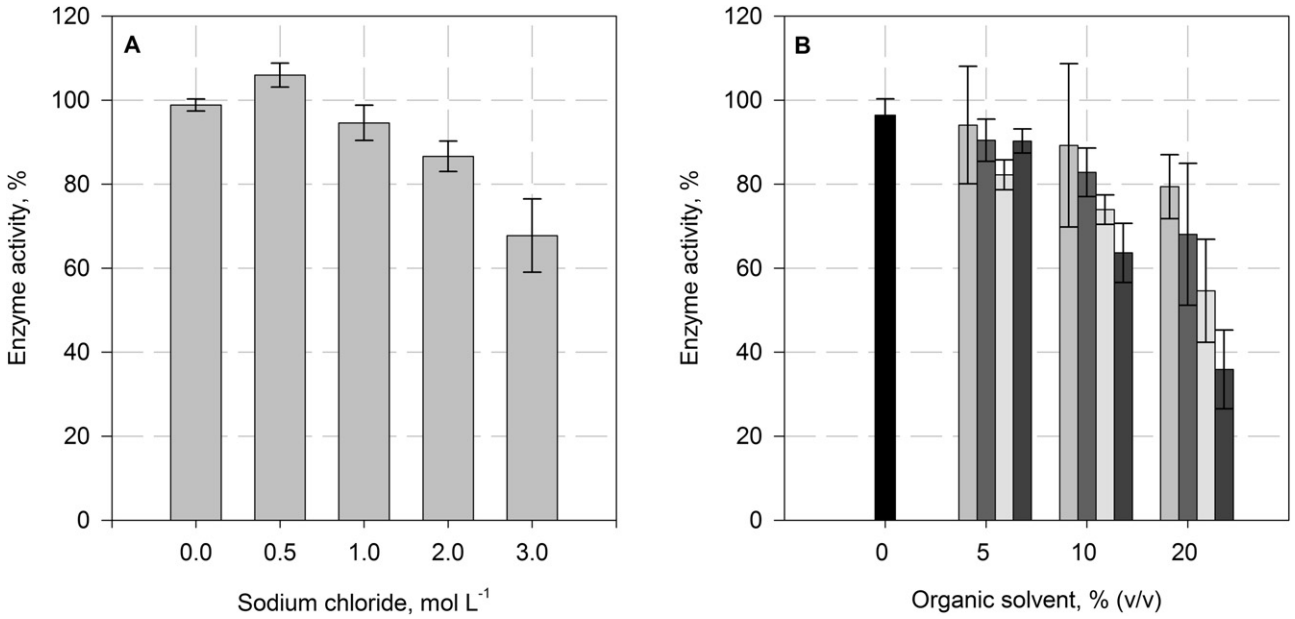


Fig. 4. Effect of salts and organic solvents on the activity of Syn7942ER. (A) Dependence of the enzyme activity of Syn7942ER on 0–3 M sodium chloride concentration. (B) Impact of 0–20% (v/v) organic solvents on the enzyme activity of Syn7942ER: reference (■), ethanol (■), 1-propanol (■), iso-propanol (■), and dimethylformamide (■). Specific activities were determined using 10 mM maleimide and 0.5 mM NADPH.

functional impairments due to a C-terminal tag has been observed for Yqjm from *Bacillus subtilis* of the dimeric "thermophilic-like" group [47], which is based on the role of highly conserved C-terminal residues in the formation of the active site and dimer–dimer interfaces [48]. In case of PETNR from *Enterobacter cloacae* st. PB2, a member of the monomeric "classical" group, in which Syn7942ER can also be assigned to, a C-terminal tag did not affect the kinetic properties at all [49]. Consequently, the effect of C-terminal affinity tags cannot be predicted based on the structural differences of the two proposed groups.

The current commercial interest on ERs has been derived from their ability of generating industrial relevant compounds with high optical purities [11]. Syn7942ER showed moderate enzyme activities using maleimides (1.35–2.30 U mg^{-1}) and enones

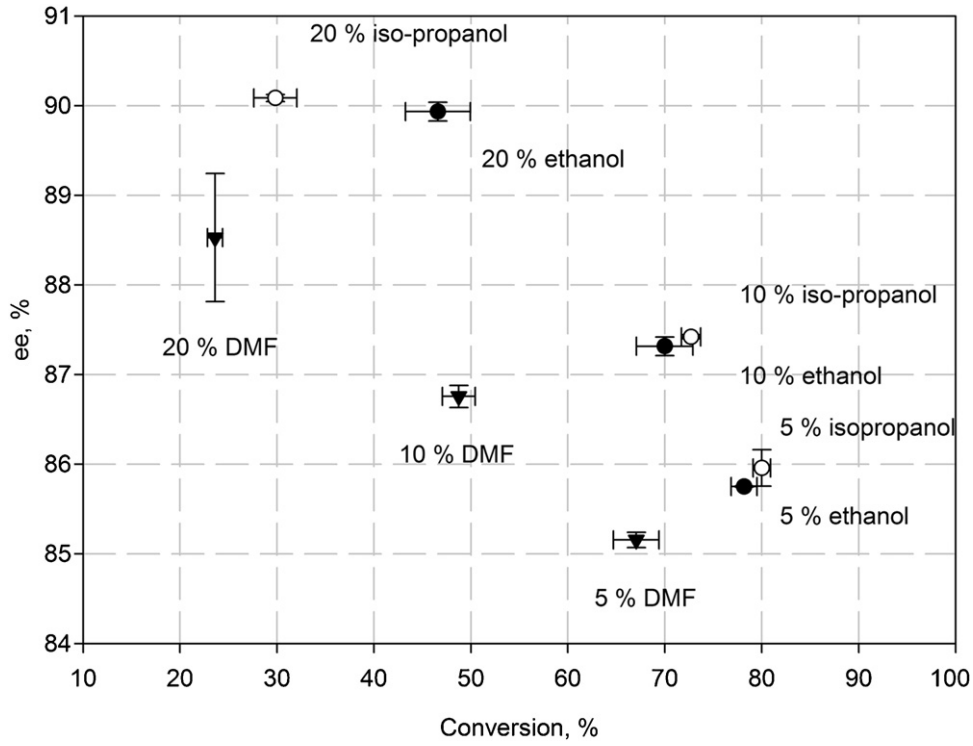


Fig. 5. Bioreduction of ketoisophorone by Syn7942ER with 5%, 10% and 20% (v/v) co-solvents: ethanol (black circle), iso-propanol (white circle) and dimethylformamide (black triangle). Reactions were performed in mL-scale with 57 μg mL^{-1} (1.2 μM) $NADP^+$-dependent *Myc*FDH C145S/D221Q/C255V, 250 mM sodium formate, 0.5 mM $NADP^+$, 10 mM ketoisophorone and 25 μg mL^{-1} (0.6 μM) Syn7942ER for 6 h at 30 °C and 150 rpm. Conversions and enantiomeric excesses (ee) were determined by chiral GC.

(0.26–1.16 U mg^{-1}), which are highly valuable building blocks for polymerization processes or synthons for pharmaceutical drug synthesis [3]. Ketoisophorone, an important intermediate for the production of carotenoids, such as zeaxanthin and xanthoxin [50], was converted with a reaction rate of 1.16 U mg^{-1}. The reduction of carvones followed reaction rates in the range of 0.37–0.61 U mg^{-1} yielding dihydrocarvones, that can be used for the synthesis of shape memory polyesters [51], antimalarial drugs and natural products [11]. In case of (R)-carvone and ketoisophorone, the activities are comparable to those of KYE from *Kluyveromyces lactis* ATCC 8585 and XenA from *Pseudomonas putida* [19].

The notable similarity of homologous ERs regarding the stereochemical outcome of the products [3] was also observed for the novel Syn7942ER. The reduction of ketoisophorone, 2-methylmaleimide and (R)-carvone furnished (R)-enantiomers. (S)-products were derived in case of 2-methyl-2-cyclopenten-1-one and 3-methyl-2-cyclohexen-1-one, which is similar to the enantiopreference of PETNR [17], OYE1-3 [26] or NemR [25]. Syn7942ER reduced 2-methylmaleimide with a high enantiomeric excess of >99% ee, consistent with published data from other ERs [4,17,25,26]. Low enantiomeric purities resulted from the reduction of 2-methyl-2-cyclopenten-1-one. This has been also observed for other ERs and explained by a flipped docking mode due to the smaller ring size of the molecule [17,52]. Additionally, it is noteworthy that (2R,5R)-dihydrocarvone was produced with the highest enantiopurity of 98% de by the reduction of (R)-carvone using Syn7942ER compared to published ERs from the literature (16% de — 97% de for PETNR [17,53], MorR from *P. putida* M10, EBP1 from *Candida albicans*, NemR [25] and wild type OYE1 [54]). Only a mutant OYE1 showed the same diastereomeric excess of 98% de [54].

Compared to the catalytic properties of *Synechococcus* sp. PCC 7942 as a whole-cell biocatalyst, similar preferences of substrate types were observed [32,33]. In whole-cell biotransformations, the cyanobacterium showed high conversion towards maleimide derivatives and α-methylated enones, whereas β-substituted enones led to low reaction rates [32,33]. Similar to the reactions with isolated Syn7942ER, (R)-enantiomers were obtained with >99% ee using maleimide derivatives [33]. In contrast to Syn7942ER, which reduced (R)-carvone and ketoisophorone furnishing (R)-enantiomers, corresponding (S)-enantiomers were produced during whole-cell biotransformations [32]. Hence, other enzymes besides Syn7942ER might be involved in the reduction of activated alkenes in *Synechococcus* sp. PCC 7942.

In this study, we have presented a new efficient biocatalyst for the C=C double bond reduction of activated alkenes. A novel ene-reductase from *Synechococcus* sp. PCC 7942 was characterized with regard to central aspects: substrate spectrum, cofactor preference, stereoselectivity and biochemical properties. Its ability to reduce a variety of activated alkenes in a highly stereoselective manner points out the potential of cyanobacterial ene-reductases for asymmetric reductions.

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References

[1] Adams JP, Collis AJ, Henderson RK, Sutton PW. Biotransformations in small-molecule pharmaceutical development. In: Whittall J, Sutton PW, editors. Practical methods for biocatalysis and biotransformations. Chichester: John Wiley & Sons; 2009. p. 1–82.
[2] Pollard DJ, Woodley JM. Biocatalysis for pharmaceutical intermediates: the future is now. Trends Biotechnol 2007;25:66–73.
[3] Toogood HS, Gardiner JM, Scrutton NS. Biocatalytic reductions and chemical versatility of the old yellow enzyme family of flavoprotein oxidoreductases. ChemCatChem 2010;2:892–914.
[4] Hall M, Stueckler C, Ehammer H, Pointner E, Oberdorfer G, Gruber K, et al. Asymmetric bioreduction of C=C bonds using enoate reductases OPR1, OPR3 and YqjM: enzyme-based stereocontrol. Adv Synth Catal 2008;350:411–8.
[5] Brenna E, Gatti FG, Monti D, Parmeggiani F, Serra S. Stereochemical outcome of the biocatalysed reduction of activated tetrasubstituted olefins by old yellow enzymes 1–3. Adv Synth Catal 2012;354:105–12.
[6] Noyori R. Asymmetric catalysis: science and opportunities (nobel lecture). Angew Chem Int Ed 2002;41:2008–22.
[7] Knowles WS. Asymmetric hydrogenations (nobel lecture). Angew Chem Int Ed 2002;41:1999–2007.
[8] Stuermer R, Hauer B, Hall M, Faber K. Asymmetric bioreduction of activated C=C bonds using enoate reductases from the old yellow enzyme family. Curr Opin Chem Biol 2007;11:203–13.
[9] Hollmann F, Arends IWCE, Holtmann D. Enzymatic reductions for the chemist. Green Chem 2011;13:2285.
[10] Meyer HP. Sustainability biotechnology. Org Process Res Dev 2011;15:180–8.
[11] Winkler CK, Tasnadi G, Clay D, Hall M, Faber K. Asymmetric bioreduction of activated alkenes to industrially relevant optically active compounds. J Biotechnol 2012. http://dx.doi.org/10.1016/j.jbiotec.2012.03.023, in press.
[12] Breithaupt C, Kurzbauer R, Schaller F, Stintzi A, Schaller A, Huber R, et al. Structural basis of substrate specificity of plant 12-oxophytodienoate reductases. J Mol Biol 2009;392:1266–77.
[13] Xu D, Kohli RM, Massey V. The role of threonine 37 in flavin reactivity of the old yellow enzyme. Proc Natl Acad Sci U S A 1999;96:3556–61.
[14] Kohli RM, Massey V. The oxidative half-reaction of old yellow enzyme—the role of tyrosine 196. J Biol Chem 1998;273:32763–70.
[15] Warburg O, Christian W. Über das gelbe Ferment und seine Wirkung. Biochem Z 1933;266:377–411 [in German].
[16] Adalbjörnsson BV, Toogood HS, Fryszkowska A, Pudney CR, Jowitt TA, Leys D, et al. Biocatalysis with thermostable enzymes: structure and properties of a thermophilic 'ene'-reductase related to old yellow enzyme. Chembiochem 2010;11:197–207.
[17] Fryszkowska A, Toogood H, Sakuma M, Gardiner JM, Stephens GM, Scrutton NS. Asymmetric reduction of activated alkenes by pentaerythritol tetranitrate reductase: specificity and control of stereochemical outcome by reaction optimisation. Adv Synth Catal 2009;351:2976–90.
[18] Richter N, Groger H, Hummel W. Asymmetric reduction of activated alkenes using an enoate reductase from *Gluconobacter oxydans*. Appl Microbiol Biotechnol 2011;89:79–89.
[19] Chaparro-Riggers JF, Rogers TA, Vazquez-Figueroa E, Polizzi KM, Bommarius AS. Comparison of three enoate reductases and their potential use for biotransformations. Adv Synth Catal 2007;349:1521–31.
[20] Kataoka M, Kotaka A, Thiwthong R, Wada M, Nakamori S, Shimizu S. Cloning and overexpression of the old yellow enzyme gene of *Candida macedoniensis*, and its application to the production of a chiral compound. J Biotechnol 2004;114:1–9.
[21] Strassner J, Furholz A, Macheroux P, Amrhein N, Schaller A. A homolog of old yellow enzyme in tomato. Spectral properties and substrate specificity of the recombinant protein. J Biol Chem 1999;274:35067–73.
[22] Blehert DS, Fox BG, Chambliss GH. Cloning and sequence analysis of two Pseudomonas flavoprotein xenobiotic reductases. J Bacteriol 1999;181:6254–63.
[23] Muller A, Hauer B, Rosche B. Asymmetric alkene reduction by yeast old yellow enzymes and by a novel *Zymomonas mobilis* reductase. Biotechnol Bioeng 2007;98:22–9.
[24] Williams RE, Rathbone DA, Scrutton NS, Bruce NC. Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl Environ Microbiol 2004;70:3566–74.
[25] Mueller NJ, Stueckler C, Hauer B, Baudendistel N, Housden H, Bruce NC, et al. The substrate spectra of pentaerythritol tetranitrate reductase, morphinone reductase, N-ethylmaleimide reductase and estrogen-binding protein in the asymmetric bioreduction of activated alkenes. Adv Synth Catal 2010;352:387–94.
[26] Hall M, Stueckler C, Hauer B, Stuermer R, Friedrich T, Breuer M, et al. Asymmetric bioreduction of activated C=C bonds using *Zymomonas mobilis* NCR enoate reductase and old yellow enzymes OYE 1–3 from yeasts. Eur J Org Chem 2008;2008:1511–6.
[27] Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 1979;111:1–61.
[28] Jones CS, Mayfield SP. Algae biofuels: versatility for the future of bioenergy. Curr Opin Biotechnol 2011;23:1–6.
[29] Ducat DC, Way JC, Silver PA. Engineering cyanobacteria to generate high-value products. Trends Biotechnol 2011;29:95–103.
[30] Hölsch K, Havel J, Haslbeck M, Weuster-Botz D. Identification, cloning, and characterization of a novel ketoreductase from the cyanobacterium *Synechococcus* sp. strain PCC 7942. Appl Environ Microbiol 2008;74:6697–702.
[31] Hölsch K, Weuster-Botz D. New oxidoreductases from cyanobacteria: exploring nature's diversity. Enzyme Microb Technol 2010;47:228–35.

[32] Shimoda K, Kubota N, Hamada H, Kaji M, Hirata T. Asymmetric reduction of enones with *Synechococcus* sp. PCC 7942. Tetrahedron Asymm 2004;15:1677–9.
[33] Hirata T, Takarada A, Hegazy M-EF, Sato Y, Matsushima A, Kondo Y, et al. Hydrogenation of the C–C double bond of maleimides with cultured plant cells. J Mol Catal B Enzym 2005;32:131–4.
[34] Mehta NB, Phillips AP, Lui FF, Maleamic Brooks RE, Citraconamic Acids. Methyl Esters, and Imides. J Org Chem 1960;25:1012–5.
[35] Fairbanks G, Steck TL, Wallach DFH. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. Biochemistry 1971;10:2606–17.
[36] Bisswanger H. Enzyme kinetics. Weinheim: Wiley-VCH; 2008.
[37] Hoelsch K, Sührer I, Heusel M, Weuster-Botz D. Engineering of formate dehydrogenase: synergistic effect of mutations affecting cofactor specificity and chemical stability. Appl Microbiol Biotechnol 2012. http://dx.doi.org/10.1007/s00253-012-4142-9, in press.
[38] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[39] Oberdorfer G, Steinkellner G, Stueckler C, Faber K, Gruber K. Stereopreferences of old yellow enzymes: structure correlations and sequence patterns in enoate reductases. ChemCatChem 2011;3:1562–6.
[40] Brenna E, Gatti FG, Monti D, Parmeggiani F, Sacchetti A. Productivity enhancement of C=C bioreductions by coupling the in situ substrate feeding product removal technology with isolated enzymes. Chem Commun 2012;48:79–81.
[41] Bechtold M, Brenna E, Femmer C, Gatti FG, Panke S, Parmeggiani F, et al. Biotechnological development of a practical synthesis of ethyl (S)-2-ethoxy-3-(p-methoxyphenyl)propanoate (EEHP): over 100-fold productivity increase from yeast whole cells to recombinant isolated enzymes. Org Process Res Dev 2012;16:269–76.
[42] Brenna E, Gatti FG, Monti D, Parmeggiani F, Sacchetti A. Cascade coupling of ene reductases with alcohol dehydrogenases: enantioselective reduction of prochiral unsaturated aldehydes. ChemCatChem 2012;4:653–9.
[43] Yanto Y, Winkler CK, Lohr S, Hall M, Faber K, Bommarius AS. Asymmetric bioreduction of alkenes using ene-reductases YersER and KYE1 and effects of organic solvents. Org Lett 2011;13:2540–3.
[44] Hall M, Stueckler C, Kroutil W, Macheroux P, Faber K. Asymmetric bioreduction of activated alkenes using cloned 12-oxophytodienoate reductase isoenzymes OPR-1 and OPR-3 from Lycopersicon esculentum (tomato): a striking change of stereoselectivity. Angew Chem Int Ed Engl 2007;46:3934–7.
[45] Winkler CK, Stueckler C, Mueller NJ, Pressnitz D, Faber K. Asymmetric synthesis of O-protected acyloins using enoate reductases: stereochemical control through protecting group modification. Eur J Org Chem 2010:6354–8.
[46] Stueckler C, Hall M, Ehammer H, Pointner E, Kroutil W, Macheroux P, et al. Stereocomplementary bioreduction of alpha,beta-unsaturated dicarboxylic acids and dimethyl esters using enoate reductases: enzyme- and substrate-based stereocontrol. Org Lett 2007;9:5409–11.
[47] Kitzing K, Fitzpatrick TB, Wilken C, Sawa J, Bourenkov GP, Macheroux P, et al. The 1.3 A crystal structure of the flavoprotein YqjM reveals a novel class of old yellow enzymes. J Biol Chem 2005;280:27904–13.
[48] Fitzpatrick TB, Auweter S, Kitzing K, Clausen T, Amrhein N, Macheroux P. Structural and functional impairment of an old yellow enzyme homologue upon affinity tag incorporation. Protein Express Purif 2004;36:280–91.
[49] Hulley ME, Toogood HS, Fryszkowska A, Mansell D, Stephens GM, Gardiner JM, et al. Focused directed evolution of pentaerythritol tetranitrate reductase by using automated anaerobic kinetic screening of site-saturated libraries. Chembiochem 2010;11:2433–47.
[50] Leuenberger HGW, Boguth W, Widmer E, Zell R. Synthesis of optically-active natural carotenoids and structurally related compounds. 1. Synthesis of chiral key compound (4R,6R)-4-hydroxy-2,2,6-trimethylcyclohexanone. Helv Chim Acta 1976;59:1832–49.
[51] Lowe JR, Tolman WB, Hillmyer MA. Oxidized dihydrocarvone as a renewable multifunctional monomer for the synthesis of shape memory polyesters. Biomacromolecules 2009;10:2003–8.
[52] Barna TM, Khan H, Bruce NC, Barsukov I, Scrutton NS, Moody PC. Crystal structure of pentaerythritol tetranitrate reductase: flipped binding geometries for steroid substrates in different redox states of the enzyme. J Mol Biol 2001;310:433–47.
[53] Toogood HS, Fryszkowska A, Hulley M, Sakuma M, Mansell D, Stephens GM, et al. A site-saturated mutagenesis study of pentaerythritol tetranitrate reductase reveals that residues 181 and 184 influence ligand binding, stereochemistry and reactivity. Chembiochem 2011;12:738–49.
[54] Padhi SK, Bougioukou DJ, Stewart JD. Site-saturation mutagenesis of tryptophan 116 of *Saccharomyces pastorianus* old yellow enzyme uncovers stereocomplementary variants. J Am Chem Soc 2009;131:3271–80.
[55] Corpet F. Multiple sequence alignment with hierarchical-clustering. Nucleic Acids Res 1988;16:10881–90.